

# Effects of UV light on cell cycle regulation in fission yeast

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*To the memory of my father*

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## **PUBLICATION I-II**

### **MANUSCRIPT 1**

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*This work is dedicated to the memory of my beloved father; you are always in my heart.*

Oslo, October 2007

A handwritten signature in blue ink that reads "Torje Tvegård". The signature is written in a cursive, flowing style.

# 1. Introduction

## The eukaryotic cell cycle

All organisms consist of cells, and a cell is the smallest entity that exhibits all the characteristics of life. The cell cycle is an ordered sequence of events in the life of a dividing cell, it is a cycle of cell growth, replication of the genetic material, nuclear and cytoplasmatic division. Before a cell can divide it has to grow in size, duplicate its chromosomes and separate the chromosomes for distribution between the two daughter cells. The cell cycle consists of four distinct phases that are separated in time, referred to as the G1, S, G2 and M phase (John, 1981). (1) The G1 phase is the period between the end of mitosis and the start of DNA replication. In G1, cells become primed for DNA replication in a process known as DNA licensing. (2) In S phase, DNA molecules are replicated and, consequently, the DNA content of the cell is doubled. (3) The G2 phase is the period after DNA replication and before mitosis. In G2, the cells are prepared for mitosis, proteins required for M phase are made and the cells undergo rapid growth, increasing the cell size. (4) The mitosis phase, M phase, can be divided into prophase, metaphase, anaphase and telophase. Chromosomes condense during prophase, align during metaphase, separate during anaphase and decondense during telophase. Nuclear division occurs in M phase and is followed by cytokinesis, the actual division into two new cells.

All eukaryotic cells decide in G1 whether to enter stationary phase (G0), sexually differentiate (meiosis) or commit to a new round of the mitotic cell cycle. The last point in G1 where this decision can be made is termed Start in yeast cells, and the Restriction Point in mammalian cells.

## Fission yeast, *Schizosaccharomyces pombe*, as a model organism

Understanding the regulation of how cells grow and divide is one of the most important and basic biological questions. Almost everything we know about the fundamental properties of living cells, how they grow and divide, how they express their genetic



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information, and how they use and store energy has come from the study of model organisms. Model organisms are chosen on the basis that they are relatively simple to grow and are easily amenable to experimental manipulation. This includes characteristics such as short life-cycle, techniques for genetic manipulation and non-specialist growth conditions. Fission yeast is a popular model organism and it has been particularly useful in the field of cell cycle research. Fission yeast is genetically tractable and can be maintained in the haploid or the diploid state. Classical genetic methods that allow the identification of new genes or the functional analysis of previously identified genes are all easily performed in fission yeast. In addition there is an extensive collection of molecular tools available for different studies (reviewed in Forsburg, 1999). Human cells are indeed complicated compared to simple yeast cells, but the basic cell cycle machinery is conserved throughout evolution from yeast to human. Results from model organisms are generally applicable to the cell cycles of all eukaryotes, which made it possible for researchers to elucidate many of the general mechanisms of the eukaryotic cell cycle. It is important to establish the basic principles in simple model organisms in order to understand the biology of human cells. Identifying the regulatory components of cell division in normal cells is essential for understanding how cancer cells escape this regulation.

In 1893, P. Lindner was the first to give a scientific description of the fission yeast *Schizosaccharomyces pombe*. He isolated the yeast from East African beer, and gave it the name *pombe*, which is the Swahili word for beer (Forsburg, 2005). The genus name *Schizosaccharomyces* reflects the characteristics *pombe* has in common with *Saccharomyces*, such as spore formation and fermentation capacity as well as the major difference between them, the morphology of cell division. In *S. pombe*, cell division occurs by fission into two daughters of equal size (therefore “schizo”), in contrast to *S. cerevisiae*, where division occurs by budding. Although both species are yeast, they are thought to have their last common ancestor more than 1 billion years ago (Forsburg, 2005). *S. pombe* is a non-pathogenic unicellular eukaryote (Figure 1). The cells are cylindrically shaped, 12–15 µm in length and 3–4 µm in diameter. The genome size is 13.8 Mb containing approximately 4900 genes arranged in three chromosomes (Wood et

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al., 2002). The fission yeast genome contains the smallest number of protein-coding genes yet recorded for a eukaryote organism. *S. pombe* has become a popular model organism to investigate basic biological processes. This is due to the fact that it is amenable to both classical and molecular genetic analysis, as well as to biochemical and cell biology studies. Furthermore, the sequence of the entire genome is available (Wood et al., 2002). An ever expanding array of tools and resources exist, such as a genome-wide deletion mutant library involving 4,500 deletion mutants out of total 4,900 *S. pombe* genes (<http://pombe.bioneer.co.kr/>), a collection of temperature sensitive mutants, various plasmids and expression systems. Taken together, this makes *S. pombe* a powerful experimental model system. Cell cycle analysis in *S. pombe* carried out by Paul Nurse was honoured with the Nobel prize in Medical or Physiology in 2001 (shared with T. Hartwell and T. Hunt), making *S. pombe* a little celebrity!



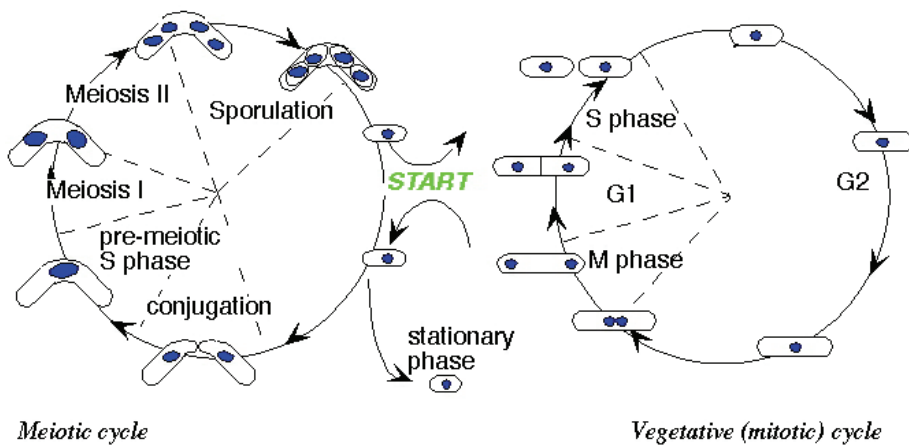
**Figure 1:** Fluorescence microscopy image of fission yeast cells where the nuclei are visualised by DAPI.

### The mitotic and meiotic cell cycles in *Schizosaccharomyces pombe*

Vegetatively growing fission yeast cells are haploid, meaning that they have one copy of every chromosome. Under standard lab conditions, *S. pombe* has discrete phases of the cell cycle much like that of higher eukaryotes except that it spends approximately 70% of its time in G2 and the three other phases occupy about 10% each. Cytokinesis is initiated at the same time as mitosis but is completed only at the end of S phase under standard laboratory growth conditions.

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*S. pombe* cells multiply asexually through the mitotic cycle or sexually through meiosis and sporulation (Figure 2). The choice between these alternative developmental pathways is linked to the nutritional status of the cell. In poor growth conditions, haploid cells of the opposite mating type will conjugate to form diploid zygotes containing two copies of the DNA. Usually, zygotes undergo meiosis immediately, followed by sporulation and formation of zygotic asci containing four haploid spores. The ascus walls may autolyse, liberating the haploid spores, which are able to survive long periods of harsh conditions. *S. pombe* cells can also be maintained in the diploid state. Diploid cells will continue mitotic growth until nutrients run out, and then they undergo meiosis and form azygotic asci, containing four haploid spores. When the environmental conditions become favourable for growth, the spores will germinate and re-enter the haploid cell cycle in G1.



**Figure 2:** Illustration of the life cycle of fission yeast. During the mitotic cell cycle one haploid yeast cell will divide into two identical daughter cells (right). The meiotic cycle is initiated in response to nutrition limitations. Two haploid cells with opposite mating type can mate, resulting in cell and nuclear fusion and formation of zygotes (left). The cells nuclei are drawn in blue. (Picture from the Forsburg lab pombe page <http://www-rcf.usc.edu/~forsburg/main.html>)

### ***Regulation of the mitotic cell cycle by the cyclin-dependent kinase Cdc2***

To ensure that each newly formed daughter cell receives a complete genome the onset and progression of S phase and mitosis are controlled so that they occur in the correct order and once in each cell cycle. Protein kinases termed **Cyclin dependent kinases** (CDKs) are the major regulators of the eukaryotic cell cycle. As the name indicates, the CDK is a kinase whose activity is dependent on a protein called cyclin. CDK and cyclin together form an enzyme that activates other proteins by phosphorylation. The amount of CDK molecules is constant during the cell cycle, but their activities vary because of the regulatory function of the cyclins (Nurse and Bissett, 1981). In addition, CDK activity may also be regulated by an inhibitory phosphorylation of the kinase and by binding to a CDK Inhibitor (CDI).

While higher eukaryotes have multiple CDKs involved in the regulation of the cell cycle, a single CDK, encoded by *cdc2*, regulates both entry into S and M phase in *S. pombe*. Cell cycle transitions are governed by fluctuations of cyclin dependent kinase activity as described below (reviewed in Moser and Russell, 2000).

### ***The Cdc2 activity can be regulated in different ways***

Cdc2 associates with different cyclins at different stages in the cell cycle and phosphorylates different substrates. There are four different cyclins that Cdc2 may associate with, Cig1, Cig2, Cdc13 and Puc1 (Fisher and Nurse, 1995; Moser and Russell, 2000). Cdc13 is required for Cdc2 activity at the onset of M phase. The Cdc13 level will therefore rise from entry into S, during G2 phase and Cdc13 is quickly degraded at the end of mitosis and throughout G1 (Creanor and Mitchison, 1996; Fisher and Nurse, 1996; Hayles et al., 1994). Cig2 is the major S phase cyclin (Fisher and Nurse, 1996). Consistent with its role in promoting S phase, Cdc2/Cig2 activity peaks in G1. Cig1 and Puc1 are also G1 cyclins, but it is less clear what they do. Cig1 and Puc1 are both suggested to act in late G1 where they target the CDI, Rum1, for degradation. To achieve cell cycle regulation of the cyclins, both Cdc13 and Cig2 contain destruction box motifs that direct ubiquitination and subsequent proteolysis. Regulating cyclin levels is a major factor in regulating CDK activity and thereby the cell cycle.

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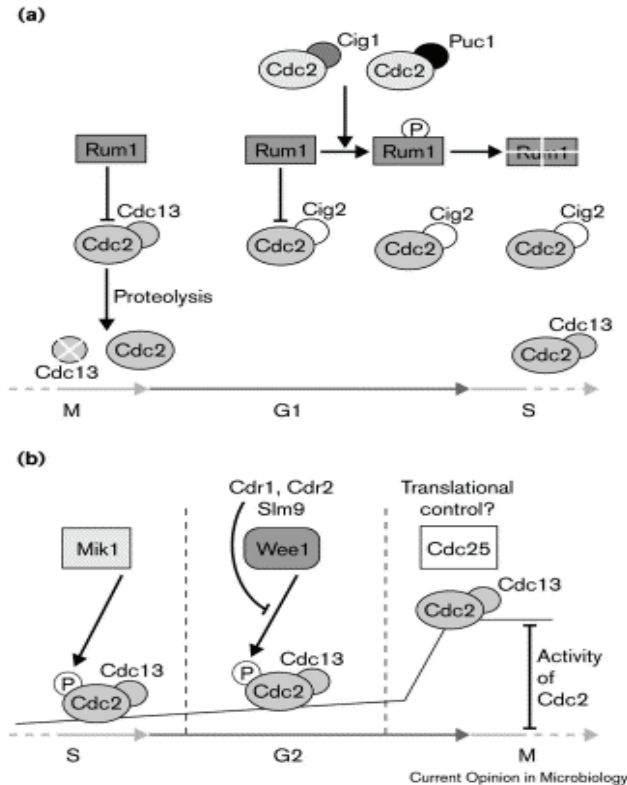
Activation of Cdc2 requires phosphorylation of Thr167 (Gould et al., 1991) and dephosphorylation of Tyr15 (Gould and Nurse, 1989). Not much is known about the significance of regulation of the phosphorylation on Thr167. In the rest of the text, when discussing Cdc2 phosphorylation, only the inhibitory phosphorylation on Tyr15 will be considered. The level of Tyr15 phosphorylation is determined by the balance between the inactivating protein kinases Mik1 and Wee1 and activating phosphatases Cdc25 and to a lesser extent, Pyp3 (Sheldrick and Carr, 1993).

CDIs are also important elements in cell cycle control, causing cell cycle delay or arrest when highly expressed, furthermore, they are typically activated in response to stresses and DNA damage. Rum1 is a CDI and is an inhibitor of S phase entry (Woollard et al., 1996). Cdc2 activity is inactivated during late M phase and G1 by Rum1. Activation of Rum1 serves to prevent entry into S phase until the events of G1 have been properly executed (Correa-Bordes et al., 1997). Phosphorylation of Rum1 in late G1 results in its degradation, allowing Cdc2/Cig2 to induce S phase entry (Moser and Russell, 2000).

There are two other proteins, Ste9 and Spd1 that have been described as regulators of Cdc2. Ste9 is required for efficient degradation of the mitotic cyclin Cdc13 during G1 arrest (Blanco et al., 2000; Yamaguchi et al., 2000). Overexpression of Spd1 (**S-phase delayed**) can arrest cells in G1 and in G2 and Spd1 can interact with Cdc2 in *in vitro* (Woollard et al, 1996). More recently it was shown that Spd1 limits dNTP levels by anchoring the small ribonucleotide reductase subunit in the nucleus (Bondar et al., 2004; Liu et al., 2003). Therefore, overexpression of Spd1 does not directly affect Cdc2 activity; the arrest in G1 is due to dNTP depletion (above) and the arrest in G2 is due to activation of a checkpoint pathway that leads to a Wee1-dependent increase of Cdc2 phosphorylation (Borgne and Nurse, 2000).

In summary, the activity of Cdc2 oscillates throughout the cell cycle (Figure 3). In early G1 there is no CDK activity and its appearance at the G1/S boundary brings about the initiation of S phase. As the cells initiate replication, phosphorylation of Cdc2 by Mik and Wee1 occurs. The CDK activity will therefore stay moderate during S phase and G2

phase. At the G2/M boundary there is an increase in CDK activity due to dephosphorylation by Cdc25, which brings about mitosis. As the cells exit mitosis, Cdc13 is degraded resulting in very low Cdc2 activity (Hayles and Nurse, 1995).



**Figure 3:** Models of cell cycle specific regulation of Cdc2. **(A)** Rum1 inhibits the Cdc2 activity throughout late M phase and G1 phase. Late in G1 phase, phosphorylation (P) targets Rum1 for degradation. In the absence of Rum1, Cdc2–Cig2 activity rises and induces entry into S phase. Cdc13 accumulates during S phase and it remains associated with Cdc2 until it is degraded upon exit from M phase. **(B)** In S phase and G2 phase, Cdc2 activity is downregulated through the inhibitory phosphorylation mediated by Mik1 and Wee1. In contrast, the Cdc2 phosphatase Cdc25 accumulates during interphase. The counterbalance of Wee1 and Cdc25 activity changes and Cdc25 is finally able to bring about Cdc2 activation, driving cells into M phase (Moser and Russell, 2000).

## Initiation of DNA Replication

In order for the genome to be faithfully maintained, chromosomal DNA must be precisely replicated and segregated in each cell cycle. Genetic analyses in simple model organisms, particularly *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Xenopus laevis*, have greatly contributed to our understanding of the regulation of DNA replication. Most factors required for initiation of replication have been identified in yeasts, and shown to be conserved in higher eukaryotes.

Initiation of DNA replication in eukaryotes is a complex and only partly characterised process (Kelly and Brown, 2000; Moser and Russell, 2000; Tabancay, Jr. and Forsburg, 2006). Initiation of DNA replication occurs simultaneously at many sites along the chromosomal DNA, and initiation of DNA synthesis is directed by specific DNA sequence elements termed replication origins. Origins are defined as cis-acting sequences that bind initiator proteins that promote early steps leading to the establishment of replication forks. These steps include unwinding of the DNA duplex, and it is from the origins that new DNA strands are replicated in both directions. The passage through start requires activation of a small number of genes, which include Cdc18 and Cdt1. The products of these genes are essential for the onset and progression of S phase and are therefore termed S-phase-specific genes. The S-phase-specific genes are activated by an essential multisubunit transcription factor called *MluI* cell cycle box binding factor (MBF). The major components of this factor are the Cdc10, Res1, Res2 and Rep2 proteins, which form different complexes. The MBF complexes bind to the *cis*-element, called *MluI* box (MCB), contained in the promoters of the S-phase-specific genes and activate the transcription of these genes.

Replication origins are marked when ORC (origin recognition complex) recognize and bind to the origins in a complex process involving six ORC subunits named Orc1- 6. ORC provides a platform for the association of essential replication factors needed to bring about initiation of DNA replication. Hundreds of origins of DNA replication are activated in order to replicate *S. pombe*'s entire genome. In budding yeast, initiation of

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DNA synthesis is directed by specific DNA sequence elements called ARS (autonomously replicating sequences) and the fission yeast origins of replication are sometimes referred to as ARS. No single conserved sequence has been identified within origins that could be an ORC-binding site in *S. pombe*, but AT rich segments seems to be important for origin function. The formation of stable complexes between ORC and origins of replication requires ATP. In ORC-DNA complexes, the bound ATP is hydrolysed only very slowly, suggesting that hydrolysis is normally coupled to some subsequent step(s) in the initiation reaction, e.g., the recruitment of additional initiator factors or DNA unwinding.

### ***Formation of the pre-replicative complex at origins of replication***

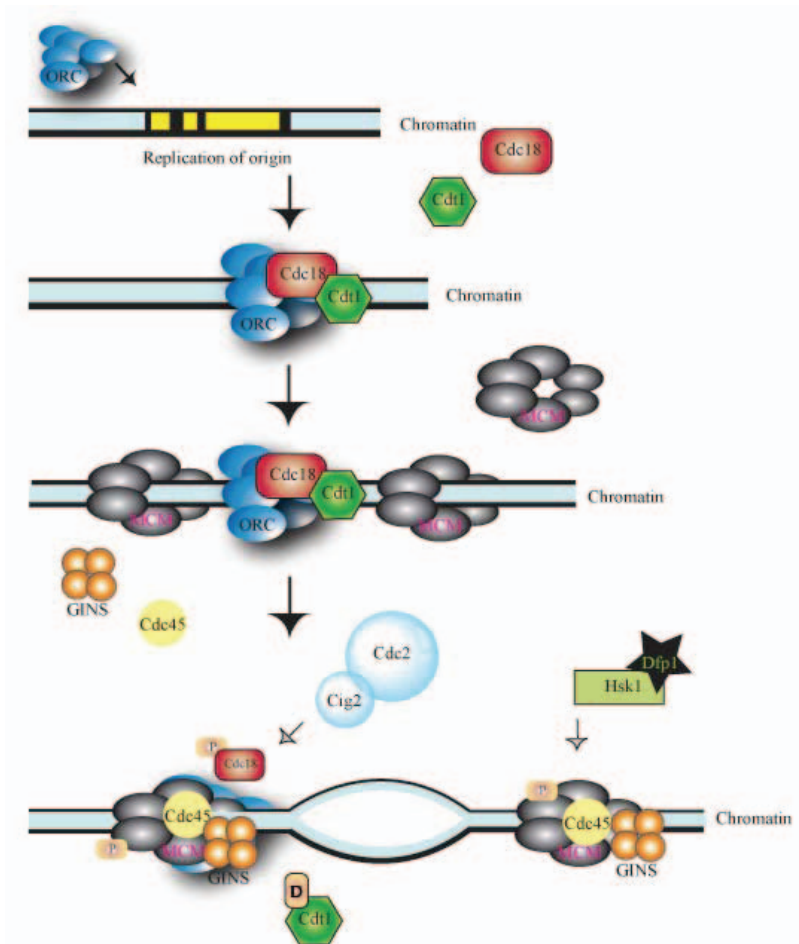
In late M phase, Cdc18 (cell division cycle 18) and Cdt1 (cdc10-dependent transcript 1) are recruited to the origin-bound ORC (figure 4). Cdc18 is required to initiate DNA replication, but it is not essential for ongoing DNA synthesis. Cdc18 and Cdt1 expression is stringently regulated in the cell cycle, through regulation of transcription and protein stability. Both *cdc18* and *cdt1* are transcribed early in mitosis under the control of Cdc10/Res1/Res2/Rep2, the transcriptional complex regulating G1- to- S progression, as mentioned above. Cdc18 is phosphorylated by Cdc2 and thereby targeted for degradation. Therefore, Cdc18 degradation is linked to high Cdc2 activity. As Cdc2 activity drops at the end of mitosis, Cdc18 accumulates and associates with origin bound-ORC. Cdt1 degradation is regulated by Ddb1 and Cdt2, two components of the of a Cul4 ubiquitin ligase (Ralph et al., 2006).

Binding of Cdc18 and Cdt1 promotes the loading of MCM (mini chromosome maintenance) proteins to origins of replication, thus forming the pre-replicative complex (pre-RC) (figure 4). Six MCM proteins, named MCM2-7, are involved in the formation of pre-RC. The six MCM-s form a hexamer that has helicase activity and probably constitutes the replicative helicase. When the pre-RC is formed the chromatin is competent for a new round of replication. However, initiation of replication requires loading of several other replication factors and also the activity of two kinases and their associated cyclin partners, Cig2 /Cdc2 and Dfp1/ Hsk1. It is unclear what the essential function of Cdc2 is for initiation of DNA replication. Cdc2 phosphorylates several



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replication factors and Cdc2 activity is required to promote Cdc45 (Sna41) loading. The Hsk1/ Dfp1 activity triggers the binding of GINS and Cdc45 to the origin. The binding of GINS and Cdc45 to origin are believed to be the crucial factors that convert the MCM complex to an active DNA helicase. GINS is a complex consisting of four proteins, and it is essential for DNA unwinding activity. The consequences of Cdc45 binding is the unwinding of DNA to open a single-stranded region and allow binding of the DNA binding protein RPA and the DNA polymerases required for leading and lagging strand synthesis.



**Figure 4:** A simplified model of DNA replication initiation in *S. pombe*. ORC binds to replication of origin and recruits Cdc18, Cdt1 and the MCM protein complex onto chromatin. This assembled origin complex is called the pre-replicative complex. Cdc2 and Hsk1 activities are essential for origin firing, followed by binding of Cdc45 and GINS. At or after origin firing, Cdt1 is targeted for degradation (D) and Cdc18 is phosphorylated, resulting in dissociation of the proteins from the DNA. Finally, the origin is initiated (indicated by the bobble in the chromatin structure).

### ***Prevention of pre-RC assembly***

The firing of each origin is tightly controlled to make sure that every piece of DNA is replicated only once per cell cycle. The control mechanism must inhibit origins from firing a second time or a replication fork from going through a region of the genome that has already been replicated. Re-replication (repeated replication of the same DNA without intervening mitosis), results in cells with multiple copies of the DNA and this polyploidisation is not desirable because it can lead to genomic instability.

Proper regulation of CDK activity is critical for preventing re-replication. This is demonstrated in cells deleted for *cdc13*, where multiple rounds of DNA replication occur, without intervening mitoses (Hayles et al., 1994). There are several ways in which CDK activity prevents re-replication. As discussed above, phosphorylation of Cdc18 by Cdc2 leads to Cdc18 degradation. Since the transcription of *cdc18* by the MBF complex is switched off during S phase, Cdc18 will not be available to support reloading of the MCM proteins. In addition, displacement of the MCMs from the chromatin and phosphorylation of at least MCM2 after firing of origin also inhibits re-replication, suggesting that MCMs serve as possible targets of negative regulation by CDK activity. Loading of MCMs to make new pre-RCs can only occur once the cells have passed through mitosis where the MCMs are dephosphorylated, and then MCM proteins are allowed to reassociate with chromatin after de novo synthesis of Cdc18 and Cdt1 (Lygerou and Nurse, 2000). Regulation of Cdt1 function and rapid inactivation of Hsk1/Dfp1 through degradation of the cyclin component when the cells enter S phase also participate in preventing re-replication.

## Checkpoints

A checkpoint inhibits or delays an event of the cell cycle if an upstream event has not been completed properly or if the DNA is damaged. The delay is considered a checkpoint if the arrest is an active response that can be overridden by a mutation or by drug treatment (Hartwell and Weinert, 1989). Checkpoints delay cell cycle progression by inhibiting the basic cell cycle machinery, e.g. when a cell encounters stalled replication forks or damaged DNA, giving the cell time to solve the problem (Rhind and Russell, 1998). Most of the checkpoints act through inhibitory phosphorylation of Cdc2 to prevent mitosis, and this phosphorylation is maintained for the duration of the checkpoint arrest (O'Connell et al., 2000). Arresting the cell cycle at M phase is important in preventing the cell from dividing with damaged or incompletely replicated DNA, which can be mutagenic or fatal for the cell.

In mammalian cells, defects in cell cycle checkpoints may be responsible for mutations and genetic instability, which are hallmarks of cancer cells (Sheldrick and Carr, 1993). It is estimated that more than one in three Norwegians will get cancer at some point in their lives ([http://www.kreftforeningen.no/om\\_kreft/hva\\_er\\_kreft/hva\\_er\\_kreft\\_2731](http://www.kreftforeningen.no/om_kreft/hva_er_kreft/hva_er_kreft_2731)). The cancer rate is thought to increase in the next decade. An understanding of checkpoints is important in the fight against cancer.

### *The four major checkpoints*

Four major checkpoints have been identified in *S. pombe*:

- 1) The G2/M checkpoint which delays mitosis in response to DNA damage in G2.
- 2) The S/M checkpoint which prevents mitosis until DNA replication has been completed.
- 3) The Intra-S checkpoint which inhibits S phase progression when the DNA is damaged.
- 4) The G1/M checkpoint, which prevent mitosis in cells which are arrested in the G1 phase of the cycle.

The four checkpoints have several features in common. First, all of them require six proteins encoded by the checkpoint *rad* genes. Second, they are all mediated by one or

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both of the two proteins kinases Chk1 or Cds1. Finally, their downstream target is the cell cycle kinase Cdc2, which is inactivated by phosphorylation on tyrosine-15 when the checkpoint is induced. Checkpoint responses in general involve the following elements: sensors (eg. the Rad proteins), adapters (Crb2 and Mrc1), effectors (Chk1 and Cds1) and targets (Cdc2). These proteins will be introduced in more detail below (reviewed in Carr, 1995;Cortez et al., 2001;Melo and Toczyski, 2002;Murakami and Nurse, 2000;O'Connell et al., 2000;Rhind and Russell, 1998;Rhind and Russell, 2000;Zhou and Elledge, 2000).

### ***Sensors of DNA damage: Checkpoint Rad proteins***

The checkpoint *rad* genes include *rad3*, *rad26*, *rad9*, *rad1*, *hus1* and *rad17*. The checkpoint mutants are highly sensitive to UV light, hence the name, Rad proteins (**r**adiation sensitive). The phenotypes of the different checkpoint *rad* mutants, including sensitivity to DNA damaging agents and lack of checkpoint response are almost identical. Rad3 plays a central role in the checkpoint signalling pathway, and activation leads to phosphorylation of the downstream effector kinases Chk1 and Cds1. Rad3 is a member of the phosphatidylinositol 3-kinase superfamily of protein kinases (PI3-kinases) which includes the mammalian **DNA**-dependent **protein kinase** (DNA-PK) that binds, and is activated by, broken DNA ends. The PI3-kinases, ATM (**a**taxia-**t**elangiectasia-**m**utated) and ATR (**A**TM and **R**ad3 related) are involved in the mammalian checkpoint responses, where ATM responds predominately to double strand breaks and ATR responds to bulky DNA lesions and stalled replication forks. The *S. pombe* homologue of ATR is Rad3 and the homologue of ATM is called Tel1. Tel1 has not been shown to be required for any checkpoint activity in *S.pombe*, but it is important for telomere maintenance.

Rad3 forms a complex with Rad26 and the association is essential for kinase activity. The Rad3-Rad26 complex is homologous to budding yeast Mec1-Lcd1 and mammalian ATR-ATRIP. Rad3 forms a complex with Rad26 independently of DNA damage or other checkpoint proteins (Kondo et al., 2001).

Rad1, Rad9, and Hus1 form a trimeric complex (the so-called 9-1-1 complex) which is structurally similar to and shows limited sequence homology to **p**roliferating **c**ell **n**uclear **a**ntigen (PCNA) of mammalian cells. PCNA is a sliding clamp that encircles DNA and

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binds DNA polymerase $\delta$ , thereby increasing the processivity of the polymerase by tethering it to its template. The 9-1-1 complex is therefore referred to as the checkpoint sliding clamp. The 9-1-1 complex might have a role in DNA repair. Rad1 is structurally similar to the Rec1 exonucleases, and the putative human homologue has nuclease activity.

Another checkpoint protein, Rad17 shows similarity to replication factor C (RFC). During replication, the RFC complex binds to DNA and recruits PCNA to the DNA template. In the DNA damaged or stalled replication fork response, Rad17 may mediate interactions with the 9-1-1 complex, actively loading the complex onto the DNA at the damaged sites or at stalled replication forks. The similarities of the checkpoint proteins to the DNA replication proteins indicate that these proteins might be involved in recruiting other factors involved in the activation of the checkpoint or DNA repair.

### ***Recognition of DNA damage by the Rad proteins***

It is not known in great detail how DNA damage is signalled to the rest of the cell and, in particular, to the checkpoint machinery. Different DNA-damaging agents generate a wide spectrum of different forms of DNA lesions, and it is unlikely that there are specific recognition mechanisms for each individual lesion.

The checkpoint proteins, in particular Rad26 and Rad17, may be able to recognise the DNA damage directly. It is indeed shown that they can localize to sites of DNA damage independently of one another (Harrison and Haber, 2006). It is possible that the repair machinery has to recognise the damage and that the checkpoint proteins are recruited to the sites of repair, either by the repair complexes themselves or by some signal that arises during processing of the damage. There is good evidence that double-strand breaks in the DNA are recognised only when, during their repair, single-stranded DNA (ssDNA) regions are bound by Replication protein A, RPA. ssDNA is a useful common checkpoint signal as it is formed during repair and at stalled replication forks. It may be hypothesized that RPA bind ssDNA in the presence of other types of DNA damage to evoke a checkpoint response. A corollary of this model is that many processes of repairing different DNA lesions have this intermediate in common and that DNA repair is

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therefore required to signal DNA damage. It has also been shown that budding yeast cells deficient in early steps of nucleotide excision repair cannot activate MEC1, the budding yeast homologue of Rad3 (Giannattasio et al., 2004) consistent with the model that damage processing, carried out by repair factors, is required for recruiting checkpoint proteins to damaged DNA.

### ***Effectors and adaptors downstream of the checkpoint Rad proteins***

Downstream of the Rad checkpoint proteins are the effector kinases, Chk1 and Cds1, which are important in transducing the checkpoint signal from the Rad proteins to the cell cycle machinery. Rad3 phosphorylates one of these two kinases when a checkpoint is activated. Cds1 is activated if the cells have replication problems or if the DNA is damaged in S phase, while Chk1 is required for cell cycle arrest in response to DNA damage in G2. Chk1 needs to associate with Crb2 and Cut5 to be able to generate the signal for cell cycle block upon damage in G2. Both Chk1 and Crb2 are phosphorylated in response to DNA damage in a checkpoint *rad* gene dependent manner. It has been shown that Crb2 is recruited to double-strand breaks independently of the 9-1-1 and Rad3-Rad26. After the initial processing of double-strand breaks, the 9-1-1 and Rad3-Rad26 complexes are required for the persistent localisation of Crb2 to the DNA lesion, which may be involved in the maintenance of checkpoint arrest during DNA repair.

The role of Cds1 is as the effector of the S-M checkpoint pathway. The Cds1 kinase is activated by inhibition of DNA replication and Cds1 is suggested to have a role in recovery from S phase arrest. Cds1 requires the adaptor protein Mrc1 for activity. Mrc1 associates with the replication machinery and thus is well placed to recruit and activate Cds1 in case of replication arrest (Gambus et al., 2006; Szyjka et al., 2005; Xu et al., 2006).

### ***The target of the checkpoints: Cdc2***

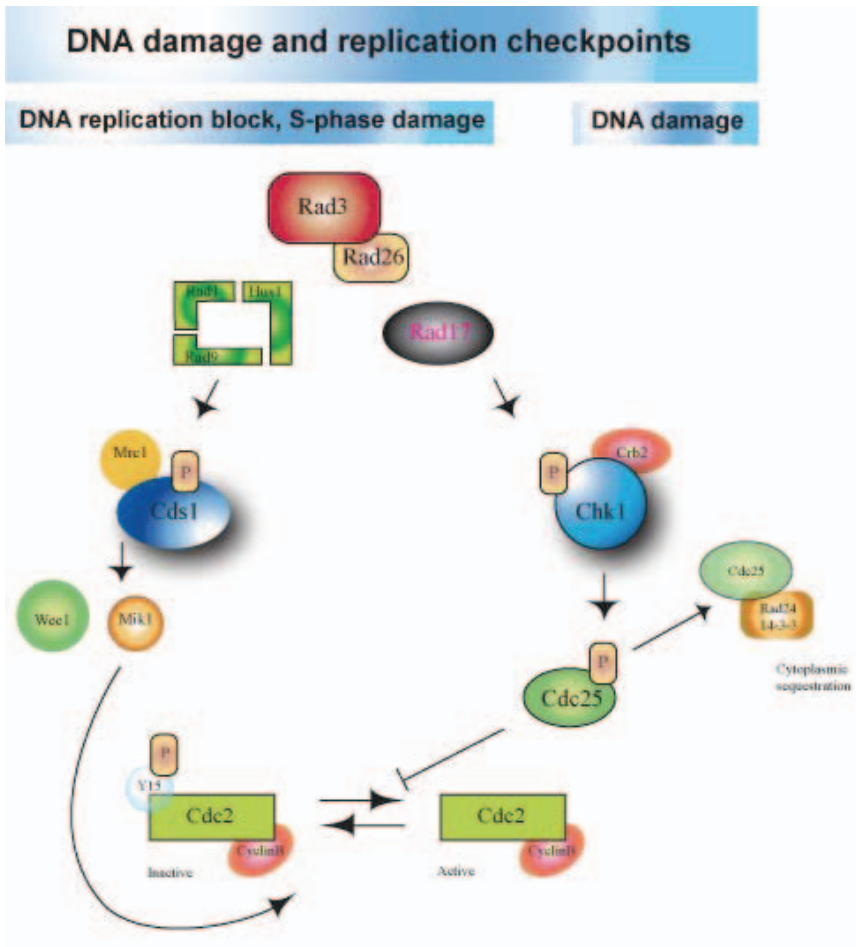
Cds1 and Chk1 inhibit entry into mitosis by increasing and maintaining the inhibitory phosphorylation of Cdc2. They achieve this by activating the kinases Mik1 and Wee1 and by inactivating the phosphatase Cdc25. The exact contribution of Wee1 in the checkpoint response is debated. Mik1 appears to be a major target of Cds1. Upregulation of Mik1 in response to DNA replication checkpoint activation is sufficient alone to arrest the cells in

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G2. Mik1 is cell cycle regulated such that it is only expressed during S phase and the S/M checkpoint maintains Mik1 expression at a high level for the duration of the arrest. Cdc25 is a major target of Chk1. Chk1 phosphorylates Cdc25 and thereby inhibits Cdc25's ability to activate Cdc2. Phosphorylation of Cdc25 by Chk1 promotes binding of the 14-3-3 proteins, Rad24 or Rad25, to Cdc25. By binding to Cdc25, they can export Cdc25 out from the nucleus.

In summary, Cdc2 phosphorylation is brought about and maintained by a combination of signals to induce the Tyr15 kinases and to inhibit the Tyr15 phosphatases.





**Figure 5:** A simplified schematic presentation of classical checkpoint responses in fission yeast. Induction of the DNA replication checkpoint pathway and DNA damage checkpoint requires a common activation of the checkpoint Rad proteins; Rad3/Rad26, Rad9/Rad1/Hus1 and Rad17. The downstream target for the classical checkpoints is the cell cycle kinase Cdc2, which is inactivated by Tyr15 phosphorylation (Y15P) when the checkpoint is induced. The Rad proteins mediate cell cycle arrest through two different kinases depending on where in the cell cycle problems are encountered. DNA replication block or S phase damage results in activation of Cds1 kinase and Cdc2 kinase activity is inhibited through phosphorylation of Tyr15 residue by Wee1 and Mik1 (left branch below the Rad proteins). DNA damage leads to Chk1 dependent phosphorylation of Cdc25, resulting in its nuclear export by Rad24 14-3-3. Nuclear export of Cdc25 inhibits activation of Cdc2 kinase (right branch below the Rad proteins).

## **Translational control**

In order for a cell to divide it has to double in mass (cell growth) as it progresses through the cell cycle in preparation for division, whereby a mother cell splits into two identical daughter cells (cell division). While cell growth of lower eukaryotes is driven by nutrient availability, higher eukaryotes need signals both from nutrients and growth factors. When nutrients and the energy status of the cells are present at sufficient levels, the cell responds by increasing the translational capacity through increased levels of ribosomes and other translational components. Ribosome biogenesis is the major consumer of energy in the cell; in an actively cycling eukaryotic cell the translational machinery occupies around 80% of nuclear transcription in yeast, and about 50% in mammalian cells (Moss and Stefanovsky, 2002).

Since cell growth depends on protein synthesis and consequently a high level of cellular energy, tight regulation of the overall protein synthesis rate in response to nutrients and energy conditions is crucial for the cell. Regulation of translation rates is not only important during cell growth, it also plays a critical role in other fundamental biological processes including development and the response to environmental stresses (Sonenberg et al., 2000).

Regulation of gene expression at the level of translation in response to stress is an important, but still not completely understood control mechanism (Holcik and Sonenberg, 2005). There is accumulating evidence that translation control plays a primary role in regulating cell cycle progression and cell differentiation (Hamanaka et al., 2005; Tvegard et al., 2007; Deng et al., 2002). Several human diseases, among them a wide range of cancers, have been linked with mutations in genes of the translational control machinery, highlighting the significance of this regulatory mechanism (Calkhoven et al., 2002; Ruggero and Pandolfi, 2003).

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The process of protein synthesis, the final step in the flow of genetic information from DNA to protein involves the sequential decoding of the mRNA into protein, performed on the ribosome. Protein synthesis is divided into three main stages: initiation, elongation, and termination.

### ***Translation initiation***

Initiation of translation is a complex process, as detailed in the brief overview below (Gebauer and Hentze, 2004; Hershey, 1991; Dever, 2002; Hinnebusch, 2000).

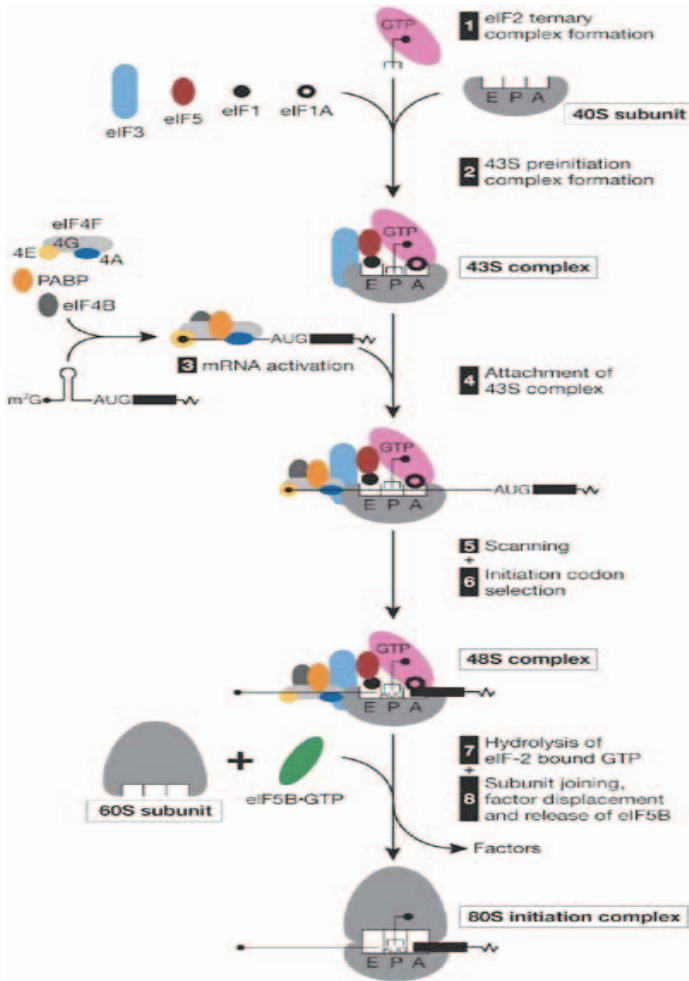
Protein synthesis is initiated when the initiator Met-tRNA<sub>i</sub> forms a ternary complex with eIF2 bound to GTP, which interacts with the 40S small ribosomal subunit and forms the 43S pre-initiation complex (see figure 6 below). A large number of proteins known as eukaryotic initiation factors (eIFs) catalyse individual steps in the initiation pathway, but only some are mentioned here.

The 43S pre-initiation complex binds to the mRNA with the help of the eIF4 group of eukaryotic translation initiation factors (eIF4F) which includes eIF4E, a cap-binding protein that is required for recruitment and binding of the mRNA to the ribosome. A special structure which consist of m<sup>7</sup>GpppN (N is any base, m<sup>7</sup>G is 7-mehtylguanylate) located at the 5' end of the mRNA is recognised by eIF4E. The structure is referred to as the 5' cap structure and it is present at the 5' terminus of all cellular eukaryotic mRNAs. eIF4A is believed to be an RNA helicase that unwinds secondary structures in the 5'UTR so that the 43S complex can bind and scan the mRNA. Yet another member of the eIF4 family is eIF4G which functions as a scaffolding protein by interacting with eIF4E, eIF4A and also the poly(A)-binding protein (PABP). In short, different eIFs working together enable the 43S pre-initiation complex to bind near the 5' cap end of the mRNA.

Once bound to the 5'cap end, the 43S pre-initiation complex migrates along the mRNA until it identifies the initiator codon AUG, forming the 48S initiation complex. Upon recognition of the initiation codon by the anticodon of tRNA<sub>i</sub><sup>Met</sup>, bound GTP is hydrolysed and eIF2-GDP is released from the ribosomal complex. The 60S subunit is

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then recruited to form the translationally competent 80S ribosome (Figure 6). The released initiation factors need to be rejuvenated for another round of translation initiation. In particular, eIF2 needs to exchange GDP for GTP.



**Figure 6:** Schematic diagram of the initiation step of protein synthesis. eIF2-GTP/Met-tRNA<sub>i</sub><sup>met</sup> ternary complex along with eIFs and a 40S subunit form the 43S pre-initiation complex. The 43S pre-initiation complex recognizes and binds to the mRNA with help of the eIF4F protein complex. The 43S pre-initiation complex together with all the subunits migrates along the mRNA until it identifies the initiator codon AUG. Stable binding of the 43S pre-initiation complex to the AUG codon yields the 48S initiation complex. Subsequent joining of the large (60S) ribosomal subunit is mediated by eIF5 and results in the formation of the 80S initiation complex. The 80S complex is capable of peptide formation. Upon AUG recognition, eIF2 and other initiator factors dissociates and are recycled for another round of initiation (not shown) (Holcik and Pestova, 2007)

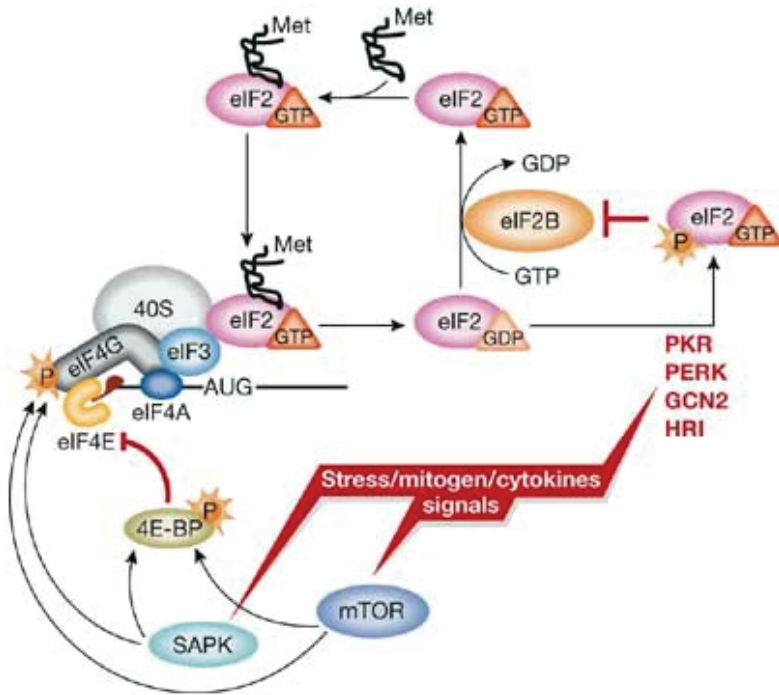
***Regulation of initiation of translation***

Although all three steps involved in protein synthesis can be subjected to regulatory mechanisms, formation of the 43S pre-initiation complex is a rate –limiting step and, as such, a well suited target for regulation. Blocking initiation is more efficient, compared to interrupting translation at later stages which would involve the need to deal with the consequences of aberrant protein synthesis.

For the ternary complex to be active, eIF2 has to bind GTP. GTP is consumed by each initiation event, leaving eIF2 bound to GDP. Therefore, reformation of the ternary complex is dependent upon restored activity of eIF2 in order to be ready for another round of initiation. Exchange of GDP to GTP on eIF2 is catalyzed by eIF2B (guanine-nucleotide-exchange factor).

Phosphorylation of the  $\alpha$  subunit of eIF2 at residue serine 51 (serine 52 in *S. pombe*) blocks the GDP-GTP exchange reaction. As a consequence, the concentration of the active ternary complex is reduced, thereby preventing the assembly of 43S pre-initiation complex, resulting in inhibition of global mRNA translation. Since there is far more eIF2 $\alpha$  than eIF2B in the cell, even small changes in the phosphorylation of eIF2 $\alpha$  can lead to significant eIF2B sequestration and will affect the translation rate.

Four kinases that phosphorylate eIF2 $\alpha$  have been identified in mammalian cells; Haem – regulated inhibitor kinase (HRI), protein kinase RNA (PKR), PKR- like endoplasmic reticulum kinase (PERK), and general control non- derepressible –2 (GCN2). The four kinases share a conserved kinase domain. However, each kinase has unique regulatory domains. These kinases are individually activated in response to different forms of stresses, but they alter protein synthesis through a common pathway, namely phosphorylation of eIF2 $\alpha$ . A simplified model of translation regulation by stress is shown in figure 7.



**Figure 7:** A simplified model of translation regulation by stress. In response to various stresses, eIF2 $\alpha$  is phosphorylated by the eIF2 $\alpha$  kinases thereby reducing the level of available ternary complex and translation of most mRNAs are down regulated. Activation of the stress-activated MAP kinase pathway (SAPK) and the TOR signaling complex disrupt the recruitment into the 48S initiation complex on the mRNA resulting in reduction of protein synthesis. (Holcik and Pestova, 2007)

## **TOR, a central controller of cell growth**

Cell growth and cell cycle progression are generally tightly coupled, but they are separable processes (Jorgensen and Tyers, 2004). Cell growth consists of synthesis of macromolecules and leads to an increase in mass or size, whereas cell division leads to an increase in cell number. Proliferation can be seen as a coordination of cell growth and division. As a major consumer of energy in the cell, mRNA translation and ribosomal biogenesis is initiated only when nutrients and growth factors (in higher eukaryotes) are available, and repressed when the supply of precursor amino acids is insufficient. However, cell growth is not passively controlled by the availability of nutrients, but by an active process involving a kinase named TOR. TOR (**t**arget of **R**apamycin) kinase is thought to sense and integrate environmental signals to control the rates of global protein synthesis and additional metabolic processes, which in turn is crucial for proper control of cell growth and cell cycle progression (reviewed in Wullschleger et al., 2006; Hay and Sonenberg, 2004). TORs are large serine/threonine protein kinases that belong to a family of phosphoinositide (PI) 3- kinase-related kinases (PIKKs; which also include ATM and ATR). TOR homologues have been found in all eukaryotic genomes. Many of the targets of TOR have been described, but less is known about the upstream events leading to TOR activation. Therefore, a good model system would greatly facilitate this line of research. In higher eukaryotes there is only one TOR gene, whereas both fission and budding yeast genome encodes two TOR proteins, Tor1 and Tor2. In fission yeast *tor1* is a non-essential gene and *tor1Δ* cells are sterile, whereas *tor2* is essential for growth (reviewed in Weisman, 2004). Whether *S. pombe* can serve as a good model system to gain further insight into the functions of TOR remains to be answered.

Not much is known about the Tor complexes, nor the cellular roles of the Tor proteins, its substrates or molecular pathways in fission yeast. Research up to date has mainly focused on whether the TOR signaling complex is conserved in *S. pombe*. Below is a brief overview of how TOR is activated and what its targets are (Jacinto and Hall, 2003). Most studies have been carried out in budding yeast and mammalian cells.



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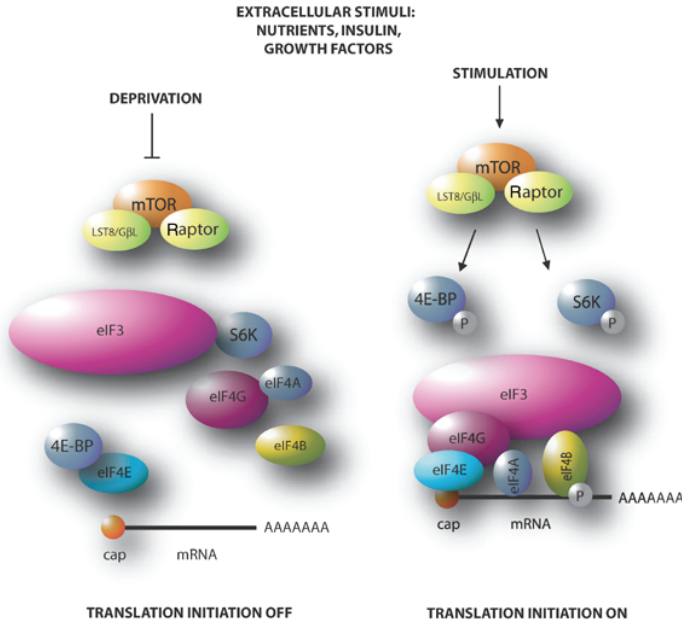
TOR interacts with a number of protein partners, including proteins that act like adapters or scaffolds, and others that regulate TOR in order to form two distinct, multiprotein signalling complexes. The two different TOR complexes are termed TOR complex 1 (TORC1) and TORC2, respectively. The TOR complexes have different roles, TORC1 mediating effects on protein synthesis and cell growth, and TORC2 affecting actin cytoskeleton and cell survival. Little is known about the TOR complexes in *S. pombe*, however recent results suggest the presence of two TOR complexes in fission yeast (Alvarez and Moreno, 2006) and the mode of action seem to be conserved from yeast to humans.

When growth conditions are favourable TOR is active and the cells maintain a robust rate of ribosome biogenesis, translation initiation and nutrient import. However, under conditions where TOR is inactive the general protein synthesis is downregulated, and several stress-responsive transcription factors are activated. TOR is inactive when nutrients are insufficient, or the cells are treated with rapamycin, or the *TOR* genes are mutated. TOR controls protein synthesis through a number of downstream targets. Some of the targets are phosphorylated directly by TOR, but many are phosphorylated indirectly. The two best characterised targets of TOR in mammalian cells are the two ribosomal S6 Kinases (S6K) and the eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP), an inhibitor of the mRNA-cap binding protein, eIF4E (figure 8). Both eIF4E and eIF4G are required for ribosome binding to mRNA (described above, section Translation) and the interaction of these two proteins is regulated by 4E-BP1. 4E-BP1 binds to the same region of eIF4E as eIF4G does, so binding of 4E-BPs to eIF4E prevents eIF4E from binding eIF4G and engaging in active translation initiation complexes. Consequently, 4E-BP behaves as a repressor of mRNA translation. Non-phosphorylated 4E-BP1 binds tightly to the translation initiation factor eIF4E, preventing it from binding to 5'-capped mRNAs and recruiting them to the ribosomal initiation complex. Upon phosphorylation by TOR, 4E-BP1 releases eIF4E, allowing it to perform its function. In budding yeast, the target of Tor is eIF4E.

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Active S6K can stimulate the initiation of protein synthesis through activation of a component of the ribosome, and other components of the translational machinery. One model for how S6K is functioning is that active S6K promotes the increased translation of 5'TOP (terminal ologopyrimidine tract) mRNAs. These mRNAs encodes exclusively components of the translation machinery, including all ribosomal proteins, elongation factors, and poly(A)-binding protein. The effect on S6K on mRNA translation is indirect via intermediates that are direct downstream effectors of S6K. Another suggested connection between the TOR/S6K pathway and translation initiation is that in the absence of extracellular stimuli such as growth factors and nutrients, S6K is associated with eIF3 preventing the formation of the initiation complex (Figure 8). Upon addition of growth factors or nutrients, TOR is recruited to eIF3 to phosphorylate S6K and 4E-BP (Figure 8). Phosphorylation of S6K leads to its dissociation from eIF3. The fully activated S6K then phosphorylates eIF4BP. Phosphorylation of eIF4B by S6K promotes its association with eIF3. All of these molecular interactions cooperates to enhance translation (Mamane et al., 2006)

TOR is activated by Rheb, a member of the Ras superfamily GTPase. Rheb is negatively regulated by the TSC1/TSC2 complex that acts as a GTPase activating protein for Rheb. Mutations in *TSC1* or *TSC2* gene cause tuberous sclerosis, a genetic disorder associated with the appearance of tumours in various organs. The *tsc1*<sup>+</sup> and *tsc2*<sup>+</sup> genes analogues to the mammalian TSC genes are present in fission yeast and their gene products form a complex which functions as a downregulator of Rheb GTPase. The homologues of the TSC genes are not found in budding yeast, and therefore *S. pombe* emerges as a promising model system in which to study upstream events leading to TOR activation (Aspuria et al., 2007)



**Figure 8:** Activation of translation initiation by mammalian TOR (mTOR). Nutrients, hormones and growth factors activate the mTOR, which forms a complex with other proteins. mTOR phosphorylates two major targets: 4E-BPs and S6Ks. Phosphorylated 4E-BP is released from eIF4E. In the absence of extracellular stimuli, S6K is associated with eIF3 and there is no initiation of translation. In response to extracellular stimuli, such as growth factors or nutrients, the mTOR complex is recruited to eIF3 to phosphorylate S6K and 4E-BP1. Phosphorylation and activation of S6K leads to its dissociation from eIF3. Activated S6K then phosphorylates eIF4B. Phosphorylation of eIF4B promotes its association with eIF3 allowing translation initiation (picture from Mamane et al., 2006)

In summary, TOR can be compared to a spider in its net. TOR receives cues from general metabolism and growth signals, just as the spider receives signals from anything touching its net. Pulling the threads in the huge intracellular regulatory network adjusts the activity of TOR and thereby all aspects of gene expression, including transcription, translation, and protein stability.

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## 2. General introduction to the projects

Several checkpoint pathways monitor the status of the DNA and arrest the cell cycle in response to DNA damage or inhibition of DNA replication in eukaryotic cells. They include mechanism to inhibit mitosis when the DNA is damaged (the G2/M checkpoint), or S phase is perturbed (the S/M checkpoint) as well as mechanism to inhibit the ongoing DNA replication when the DNA is damaged (the intra-S checkpoint) and mechanisms that inhibit entry into S phase when the DNA is damaged in G1 (G1/S checkpoints). An important feature of cancer cells is an escape from the checkpoint arrests; therefore, an understanding of checkpoint regulation is important. Fission yeast offers several experimental advantages to investigate checkpoint pathways and such studies have greatly contributed to our current understanding of the S/M and G2/M DNA damage pathways. These checkpoint pathways are evolutionary conserved from yeast to man, underlining their importance in maintaining the genome integrity.

G1 is a very important phase in the cell cycle because it is in G1 that the cells decide whether to proceed into another mitotic cell cycle, enter the meiotic cell cycle, or enter stationary phase. To make the right decision in G1 is critical for the cells, since alterations in the regulation of the G1/S transition will lead to genetic instability and cancer development.

Three checkpoints have been shown to act in G1 in fission yeast, and they all work to inhibit mitosis (Carr et al., 1995; Hayles and Nurse, 1995; Synnes et al., 2002). Apart from those studies, checkpoints in G1 phase of *S. pombe* have not been extensively investigated. The main reason for this may be that *S. pombe* cells have an extremely short G1 phase under normal laboratory growth conditions, making it difficult to investigate G1 events.

In 2001, when I was a *cand. scient* student I joined Erik Boye's lab group whose group was particularly interested in G1 events in fission yeast. The group had at that time identified and partly characterised a novel checkpoint in G1, which serves to inhibit the

## 2. General introduction to the projects

onset of S phase after UV irradiation in G1 (Nilssen et al., 2004; Nilssen et al., 2003). The mechanism of the checkpoint was at this stage unknown. All research in the lab aimed at exploring the mechanism of the UV-induced delay at S phase entry. My first project was to investigate the UV- induced delay at S phase entry in germinating fission yeast spores (see project 1 below). Most of the experimental work for this project was carried out as part of my *cand. scient* thesis. When I continued as a PhD student I did some of the final experiments to prepare this work for publication (Publication I). The major part of my PhD project was exploring the mechanism of the novel checkpoint using an alternative approach (see project 2 below). This work led to the identification of the major players of the novel checkpoint pathway and of a possible link between cell growth and the cell cycle (Publication II). Finally, as an effort to identify upstream components of the checkpoint, I investigated the involvement of the Tor complex. This led to the unexpected finding that Tor regulates translation initiation via eIF2 $\alpha$ , in addition to its previously described roles in regulating translation.

### 3. Aims of the projects

#### Project 1

The aim was to find the molecular mechanism(s) that regulates entrance into S phase upon UV irradiation in G1. My first project was to investigate the role of four different genes (*caal*, *crb2*, *mrc1* and *spdl*) which are associated with regulation of the cell cycle and were possible candidates to be involved in the cell cycle delay. An attractive approach to study G1 events was to use a natural phenomenon, namely germinating spores, because germinating spores exhibit a long G1 phase. In addition, germinating spores allowed the analysis of mutants that would not have been possible if temperature sensitive mutants had been employed to synchronise the cells in G1. This work was published in BMC Cell Biology, 2004 (Publication I).

#### Project 2

In order to explore the nature of the G1 checkpoint further the *cdc10* block-and- release approach was chosen, which gives better synchrony and is technically easier than spore germination experiments. Cdc10 mediates transcription of a set of genes in G1 and the corresponding proteins are required for the start of and progression through S phase. Therefore, *cdc10<sup>ts</sup>* cells arrest in G1 at the restrictive temperature. By shifting the temperature back to the permissive temperature, a highly synchronised population of cells is obtained. To follow progression from G1 to S phase Nilssen et al (2003) investigated the kinetics of chromatin binding of the Mcm proteins. The Mcm proteins are essential parts of the pre-replicative complex (pre-RC), a complex assembled at origin of replication making the cells competent for DNA replication. Nilssen et al found that pre-RC loading onto chromatin was delayed upon UV irradiation in G1. Pre-RC formation is stringently regulated in the cell cycle, but it had not been identified as a checkpoint target before. The aim of the main part of my work was to identify the molecular mechanism of this delayed pre-RC loading. During this work, we established a possible link between

### *3. Aims of the projects*

cell cycle progression (pre-RC formation) and cell growth (translation initiation). This work was published in *Genes and Development*, 2007 (Publication II).

### **Project 3**

The TOR pathway is known to regulate translation initiation in mammalian cells. Two Tor kinases have previously been identified in *S.pombe* and we wanted to investigate whether depression of translation upon UV irradiation requires Tor function. This work is presented as a manuscript (Manuscript 1).

## 4. Results and discussion

### Project 1

#### **Germinating fission yeast spores delay in G1 in response to UV irradiation**

Fission yeast spores are in a G0-like state with one copy of each chromosome. When the environmental conditions are favourable they germinate and proceed into G1. We exploited the natural synchrony of germinating spores to investigate the effect of UV irradiation on S phase entry and showed that germinating wild type spores delay entry into S phase after UV irradiation in G1. We investigated the involvement of the checkpoint *rad* genes and other possible genes known to be involved in cell cycle regulation, such as *rum1*, *res2*, *caa1*, *crb2*, *spd1*, *mrc1* and *res1*. Of these, only *rum1* seemed to be required for the G1 delay. However, Rum1 is a protein required for keeping the cells in G1 phase, therefore it is not clear whether Rum1 is a target of the checkpoint that leads to the delay or is simply required for a prolonged G1.

In contrast to what happens in other classical checkpoints, Cdc2 is not phosphorylated during the course of the delay. The S phase entry is delayed by a mechanism that is different from classical checkpoint responses, but how the cells delay cell cycle progression is not clear.

One possible explanation for the G1 delay is that proteins required for entry into S phase are not present. Germinating spores exposed to UV irradiation delayed with low levels of Cdt1, a protein essential for pre-RC formation. The levels of *cdt1* mRNA was not affected, as measured by northern blot analysis. A possible mechanism behind the G1/S checkpoint observed in germinating spores might be due to reduced translation or degradation of Cdt1. Since Cdt1 is present in low levels in the cells, pre-RC can not be formed; consequently the cells can not initiate DNA replication and the cells arrest in G1.

#### *4. Results and discussion*

We also found that when cells were irradiated in late G1 there was no delay at entry into S phase, but the cells arrested in S phase. The molecular mechanism of the arrest in S phase when cells are irradiated in late G1, shows all features of a classical checkpoint response. We concluded that cell cycle progression is only delayed when cells are exposed to UV irradiation in early G1; cells irradiated in late G1 are arrested by the intra-S phase checkpoint.

## Project 2

### A novel checkpoint mechanism regulating the G1/S transition

Ultraviolet irradiation of *cdc10*-arrested cells induce a delay in chromatin binding of the MCM proteins (pre-RC formation) and, consistently, a transient delay in S phase entry (Nilssen et al., 2003). We explored the possibility that activation of the transcriptional programme required for S phase entry is inhibited after UV irradiation. To this end, we compared the transcription patterns of control and UV-irradiated cells, measured by microarray analysis, and found no dramatic differences (H, Skjølberg, unpublished data). Furthermore, all known components required for pre-RC are synthesized on time even in UV-irradiated cells, suggesting that an active mechanism is inhibiting entry into S phase.

We noticed that cell growth in *cdc10<sup>ts</sup>* cells was affected by UV irradiation; growth measurement indicated slower growth for irradiated than non-irradiated cells. Since protein synthesis is required for cell growth, we measured the rate of translation in the cell and found that the general translation was severely depressed after UV irradiation. Consistent with these findings, eIF2 $\alpha$ , a key protein in the regulation of translation initiation, was found to be phosphorylated in UV-exposed cells. Gcn2 is a kinase known to phosphorylate the Serine-52 residue (Serine-51 in mammalian cells) on eIF2 $\alpha$  when the levels of nutrients are limiting (reviewed in Hinnebusch, 2000). We showed that UV irradiation activates Gcn2 which results in eIF2 $\alpha$  phosphorylation and repressed translation. Furthermore, pre-RC formation was delayed and thus the irradiated cells delayed entry into S phase. Our data suggest that the novel mechanism of the response to UV irradiation is linked to translational regulation. We have identified the major steps and players of the G1/S delay, but there are many questions that remain to be answered, as discussed in the following sections.

##### ***The novel pathway discovered in *cdc10*-arrested cells is a bona fide checkpoint***

A checkpoint is defined as a mechanism that prevents cell cycle transition until previous events have been completed or damaged DNA has been repaired. Cell cycle delay is a general response to DNA damage. Such a delay is considered a checkpoint if the arrest is an active response that can be overridden by a mutation or by drug treatment (Hartwell and Weinert, 1989). In a *gcn2* mutant, there is no reduction in the translation rate in response to UV irradiation and the UV-induced cell cycle delay is abolished. Therefore, the G1 delay we observed is totally dependent on Gcn2 and the cell cycle delay satisfies the definition of a checkpoint. In addition, caffeine can abolish the checkpoint response. These two findings confirm and establish the response as a *bona fide* checkpoint mechanism.

## **General discussion based on project 1 and project 2**

### ***Different biological systems to investigate G1 events***

The original discovery of the novel G1/S checkpoint in fission yeast by Nilssen et al (2003, 2004) was made using different methods to synchronise the cells in G1 and/or to extend G1. We induced synchrony using temperature-sensitive cell cycle mutants, namely *cdc10* and *cdc25*, and we used selection synchrony of cells growing in poor growth conditions. The G1/S delay was observed in all three experimental systems. For technical reasons and because the *cdc10* block-and-release experimental system gives a much better synchrony compared to germinating spores, most of the subsequent experiments were carried out using *cdc10<sup>ts</sup>* mutant cells. This approach has some potential drawbacks, given that we interfere with the regulation of the very cell cycle phase we are studying. Therefore, we wanted to exploit the natural synchrony of germinating spores. In spite of their relatively poor synchrony, we considered it a useful model system, since spore germination is a natural process. Furthermore, this model system allowed us to investigate the effects of a number of mutations that would not have been possible when the cells were synchronised by other methods. Investigating the response of UV irradiation in different biological system adds weight to the results



obtained and prevents incorrect conclusions based on artefacts due to the different synchronisation methods or temperature sensitive mutations used.

##### ***Delayed entry into S phase is brought about by different pathways in germinating spores and *cdc10*-arrested cells***

The inhibitory mechanisms found in UV-irradiated *cdc10*-arrested cells and in germinating spores have several features in common. In both experimental systems, the cells delay entry into S phase when irradiated in G1; both pathways are different from the classical checkpoint responses and neither of them involves the inhibitory phosphorylation of Cdc2. Rum1 expression is increased and sustained during the course of the delay in both cases. It should be noted that Rum1 is present in G1 cells, and it is required for all G1 delays and arrests described. We are unable to explore the role of Rum1 as a checkpoint target further in *cdc10<sup>ts</sup>* cells because lack of *rum1* produces a cell that is unable to arrest pre-Start and its failure to inhibit Cdc2 leads to premature mitosis (Moreno and Nurse, 1994). It is not clear whether Rum1 is a checkpoint target, or is simply needed for the extended G1.

In both systems, a delayed entry into S phase was only observed if cells were exposed to UV irradiation in early G1. If irradiated late in G1 they entered S phase with the same timing as non-irradiated cells. Irradiated cells arrested in S phase, probably due to the activation of the classical checkpoint. The definition of early and late G1 in respect to the timing of irradiation is rather vague. Given that pre-RC formation is delayed after irradiation in early G1, we conclude that cells irradiated before the pre-RC-s are formed will stop in G1, but cells irradiated after pre-RC formation will enter S phase.

The main difference between the two experimental systems is the expression of Cdt1. When germinating spores are exposed to UV irradiation, cells arrest with low levels of Cdt1. This is in contrast to the situation for *cdc10*-arrested cells, where there is no difference in the expression of Cdt1 in irradiated and non-irradiated cells. Based on these results, it is likely that the mechanisms of delaying S phase progression are different in

germinating spores and cells that are arrested at the *cde10*-arrest point and then released into the cell cycle.

It is curious that *S. pombe* cells delay S phase entry upon exposure to UV in G1 in a different mechanism in the two biological systems. One would think it is wasteful for the cell to operate with different checkpoints that respond differently to the same UV damage in the same phase of the cell cycle. Given that G1 is such an important cell cycle phase, the cells might respond differently in cycling contra non-cycling cells, or due to the different synchronisation methods they might be in different parts of G1, with different sets of proteins being expressed and/or activated.

#### ***What is the link between eIF2 $\alpha$ phosphorylation and cell cycle regulation?***

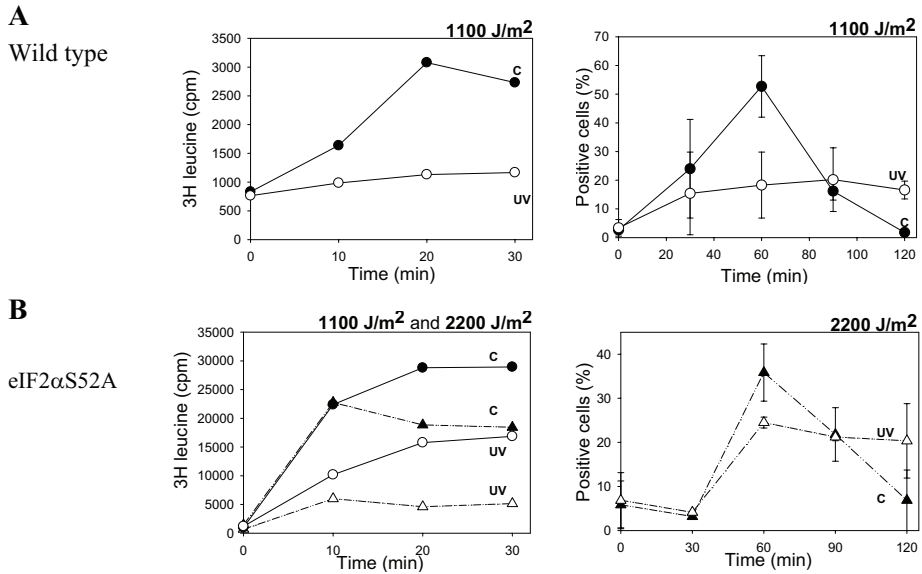
Phosphorylation of eIF2 $\alpha$  is a general cellular reaction to many forms of stress, such as UV irradiation, endoplasmic reticulum (ER) stress, activation of the unfolded protein response pathway (UPR), proteasome inhibition and apoptosis in yeast and mammalian cells (Deng et al., 2002; Hamanaka et al., 2005; Zhan et al., 2004; Hinnebusch, 2000). The phosphorylation can be mediated by several different protein kinases (Wek et al., 2006). A link between eIF2 $\alpha$  phosphorylation and cell cycle regulation has not been shown, except for human cells where the unfolded protein response (UPR) acts via inhibition of cyclin D1 mRNA translation. The cyclin D1 is an important regulator of G1 and an important cofactor for several transcription factors in numerous cell types in mammals. The inhibition of cyclin D1 synthesis in response to UPR is responsible for the induction of a G1-phase arrest (Hamanaka et al., 2005).

Phosphorylation of eIF2 $\alpha$  seems to correlate with the induction of the G1/S checkpoint in fission yeast, but the significance of this phosphorylation for cell cycle progression is not known. It has been suggested that passage through Start in budding yeast cells requires a critical rate of overall translation (reviewed in Jorgensen and Tyers, 2004). Currently, we do not know whether the cell cycle delay is directly dependent on the phosphorylation of eIF2 $\alpha$ , or whether it is an indirect effect in response to the fact that eIF2 $\alpha$  is phosphorylated. In the latter scenario, depression of translation due to eIF2 $\alpha$

phosphorylation down to a certain absolute level might trigger the cell cycle delay. In order to investigate the role of eIF2 $\alpha$  phosphorylation in the G1 checkpoint, we constructed a mutant harbouring the non-phosphorylatable form of eIF2 $\alpha$  (referred to as eIF2 $\alpha$ S52A). The eIF2 $\alpha$ S52A mutant failed to arrest the cells in G1 i.e. formation of pre-RC is not delayed, supporting the model that eIF2 $\alpha$  phosphorylation per se as the signal mediating the cell cycle delay. Considering that phosphorylation of eIF2 $\alpha$  is known to be a major regulator of translation initiation, it was a surprising finding that UV irradiation does repress translation in this mutant to some extent. These results indicate that the Gcn2 kinase can inhibit translation by a pathway distinct from that involving the known phosphorylation site of eIF2 $\alpha$  and that eIF2 $\alpha$  phosphorylation is not absolutely necessary for global translation depression when the cells are exposed to UV irradiation.

Another attempt to distinguish between whether it is depression of translation or phosphorylation of eIF2 $\alpha$  that is required for the checkpoint was to investigate whether a stronger depression of protein synthesis was required to affect pre-RC formation in the eIF2 $\alpha$ S52A mutant. We reasoned that if the overall translation rates need to be reduced to a certain level in order to induce a checkpoint response, pre-RC loading will be delayed if we can repress the translation rate in the eIF2 $\alpha$ S52A mutant down to the same level as seen in irradiated wild type cells. To examine this, the eIF2 $\alpha$ S52A mutant was exposed to a double dose of UV irradiation which gave an equally strong reduction in translation as that seen in irradiated wild type cells (figure 1A, below). In a *gcn2 $\Delta$*  mutant the double or even a triple dose neither reduced translation rate nor brought about a cell cycle delay, demonstrating that the higher dose not excessively damage components of the translation machinery. Although the protein synthesis was severely depressed in response to a double UV dose, pre-RC formation was not delayed (figure 1B, below) in the eIF2 $\alpha$ S52A mutant, further supporting the model that phosphorylation of eIF2 $\alpha$  per se is required for the cell cycle delay. However, the eIF2 $\alpha$ S52A mutant did not behave like wild type cells; the eIF2 $\alpha$ S52A mutant strain did not arrest properly in G1 at the restrictive temperature and a fairly large proportion of the cells were binuclear. Furthermore, the translation capacity of the mutant cells after double dose of UV seemed to be higher than that of wild type cells after a single dose of UV. Due to these observations, there are some

uncertainties about the interpretations of the data gained from the eIF2 $\alpha$ S52A mutant, and we can not exclude that the cell cycle delay is dependent on depression of translation down to a certain absolute level rather than on the presence of phosphorylated eIF2 $\alpha$ .



**Figure 1:** The kinetics of protein synthesis and pre-RC formation in wild type cells (**A**) and the eIF2 $\alpha$ S52A mutant (**B**). Filled symbols represent control samples, empty symbols represent UV irradiated samples. Left panels: *cdc10<sup>ts</sup>* Mcm6-GFP cells were synchronised and UV- irradiated with 1100J/m<sup>2</sup> (circles) and 2200 J/m<sup>2</sup> (triangles), respectively and released into the cell cycle. Samples were taken every 30 minutes after release to measure, in cell extracts, incorporation of 3H-leucine into TCA-perceptible material. Right panels: Percentage of cells that contained chromatin-bound Mcm6-GFP (pre-RC) at different time points after release into the cell cycle. C= control cells; UV= UV irradiated cells. Data shown are average of three independent experiments (Grallert and Boye, submitted)

Yet another alternative explanation is that it is neither eIF2 $\alpha$  phosphorylation nor translation rates but the Gcn2 kinase that links the cell cycle delay to eIF2 $\alpha$  phosphorylation and translation depression. In this model a hitherto unidentified target of Gcn2 mediates the cell cycle delay in G1.

In order to further test whether the phosphorylation of eIF2 $\alpha$  is normally required for the checkpoint we are currently constructing a mutant that mimics eIF2 $\alpha$  phosphorylation without the inducing UV treatment. We are changing the Ser52 to Asp or Glu, negatively charged amino acids that are expected to mimic phosphorylation. However, such a mutant is expected to have very low translation rates and grow very poorly. Therefore we will employ a technology that has recently been adapted for fission yeast (Bøe, C et al., in preparation). We will fuse the eIF2 $\alpha$ Ser52Asp or eIF2 $\alpha$ Ser52Glu mutants to the hormone binding domain (HBD) of the estrogen receptor (ER). The ERHBD is bound by the Hsp90-molecular chaperone and thus the fusion protein is expected to be inactive upon addition of estradiol the ERHBD is quickly released and the fusion protein is activated. Although this system does not work for all proteins, it might provide a way to express a potentially harmful eIF2 $\alpha$ Ser52”P” mutant in the cells and address whether quick activation of the mutant protein, mimicking phosphorylation, brings about a cell cycle delay

We found that the UV-induced phosphorylation of eIF2 $\alpha$  is not specific to irradiation in G1 phase, since we measured the same response in G1, in S phase and in G2. Therefore, the depression of protein synthesis might affect progression in any part of the cell cycle, in addition to the already known and characterised checkpoint responses.

#### ***How is Gcn2 activated and what is its target?***

The novel G1/S checkpoint is totally dependent upon the kinase Gcn2. How Gcn2 is activated by UV irradiation and how it mediates the checkpoint signalling is not at all understood. Some ideas about Gcn2 activation and its targets are discussed below.

The mechanism of Gcn2 activation in response to starvation is well described, especially in budding yeast. The lack of amino acids creates a problem for the aminoacyl-tRNA synthetases in recruiting amino acids to be added onto their cognate tRNAs. Uncharged tRNA will bind and activate Gcn2, resulting in phosphorylation of eIF2 $\alpha$  and inhibition of translation (reviewed in Hinnebusch, 2000).

An important task will be to identify the cellular sensor that activates Gcn2 after UV irradiation. UV light is known to generate pyrimidine dimers in DNA and the response to DNA damage has been extensively studied, but proteins, mRNA, tRNA, rRNA and fatty acids are also damaged by UV irradiation. Several lines of evidence suggest that this checkpoint is not a general DNA damage response. We have exposed the cells to various DNA damaging agents and monitored eIF2 $\alpha$  phosphorylation and G1/S delay. There is a good correlation between eIF2 $\alpha$  phosphorylation and G1/S delay, but there is no correlation between DNA damage and the G1/S delay (Krohn, M. et al, in preparation).

Data concerning the nature of how UV light is detected by the cells are not yet provided. Given that Gcn2 is activated by uncharged tRNAs in response to starvation, we are exploring the possibility that a similar mechanism might result in the activation of Gcn2 after UV light. RNA molecules can absorb UV light and, interestingly, photoreactive bases in some *Escherichia coli* tRNA-s are known to be modified by ultraviolet light such that the modified tRNA-s cannot efficiently bind their cognate amino acids, thereby leading to the accumulation of uncharged tRNA-s (Thomas et al., 1981). We are currently investigating whether UV irradiation increases the presence of uncharged tRNA in *S. pombe*.

It makes sense for the cells to inhibit translation when there is a shortage of amino acids, and it is interesting to speculate whether the cells, after suffering damage from UV irradiation, might use the same pathway to delay the cell cycle that is used for metabolic stress responses. Several types of stress elicit specific transcriptional responses, but controlling expression of the existing mRNA rather than controlling the *de novo* synthesis of mRNA might be more advantageous. The cells are able to downregulate energy-

demanding processes and arrest growth immediately by targeting initiation of translation. And the process can easily be initiated again, when the conditions are favourable.

We speculate that there are other relevant targets for Gcn2 in addition to eIF2 $\alpha$ , since in cells with the non-phosphorylatable version of eIF2 $\alpha$  translation rates are significantly reduced after UV irradiation. Even though a good target of inhibition of protein synthesis is via regulation of the initiation step, it is not unlikely that, in addition, Gcn2 phosphorylates elongation factors, or that it has some unknown substrates that regulate protein synthesis. Another possibility is that Gcn2 can phosphorylate eIF2 $\alpha$  on other sites than Serine-52, thereby affecting translation.

We have shown that the components required for pre-RC formation are present at the correct time even after UV irradiation, but it is conceivable that one or more of them are modified and/or mislocalised in the cell. It is known that in mammalian cells some of the Mcms are modified in S phase (Cortez et al., 2004; Shechter and Gautier, 2004). Upon phosphorylation, the association of Mcms with chromatin is disrupted; they are unable to participate in pre-RC formation and/or to serve as a platform for other replication factors. It is possible that Gcn2 (directly or indirectly) phosphorylates Mcms upon UV irradiation making them dissociate from the pre-RC complex, thereby delaying cell cycle progression.

Our pre-RC data do not demonstrate whether UV irradiation delays the loading of the Mcm proteins or stimulates their unloading. In the latter scenario, the pre-RCs may be formed with normal kinetics after UV exposure, but the Mcms are removed as they are loaded onto chromatin e.g. by phosphorylation (performed by Gcn2), for a limited period of time.

#### ***What is the role of Rad3 and other classical checkpoint proteins?***

Initially, Rad3 activation was shown not to be responsible for the checkpoint induction in G1, as judged by the transition from G1 to S measured by an increase in DNA content by flow cytometry (Nilssen et al., 2003, Nilssen et al., 2004). However, due to poor

#### 4. Results and discussion

resolution of the flow cytometry analyses, it was not firmly determined whether the delay in the *rad3Δ* mutant was in G1 or early S phase. I have employed the Mcm-GFP chromatin binding assay to investigate the role of Rad3 further. Interestingly, preliminary data suggest that pre-RC formation is not delayed in a *rad3Δ* mutant strain after UV irradiation. I have also monitored the phosphorylation of eIF2 $\alpha$  in cells lacking Rad3, and found that eIF2 $\alpha$  phosphorylation is strongly induced in response to UV irradiation. These preliminary data suggest that Rad3 operates downstream of Gcn2, since phosphorylation of eIF2 $\alpha$  is not dependent upon Rad3. Excitingly, these data indicate a totally novel function of Rad3, different from that operating in a classical DNA damage checkpoint. Rad3 is known to phosphorylate Mcm proteins in human cells (Cortez et al., 2004; Shechter and Gautier, 2004). Mcm could be modified by Rad3 (or Gcn2) during the checkpoint, thereby disrupting the pre-RC formation as discussed above.

It should be mentioned that making the *rad3Δ mcm6-GFP cdc10<sup>ts</sup>* strain was difficult. Only one colony was isolated which arrested properly in G1 and retained the Mcm6-GFP construct out of hundreds. All the other progeny from the appropriate cross either lost the ability to arrest in G1 or lost the GFP tag. Therefore, results obtained with the *rad3Δ mcm6-GFP cdc10<sup>ts</sup>* strain need to be confirmed in strains where Mcms other than Mcm6 are GFP-tagged or in other isolates of *rad3Δ mcm6-GFP cdc10<sup>ts</sup>*. Strain constructions of other GFP-labelled Mcm proteins in combination with *rad3Δ cdc10<sup>ts</sup>* are in progress, and as for the *rad3Δ mcm6-GFP cdc10<sup>ts</sup>* mutant strain the wanted genotypes seem hard to achieve. These difficulties indicate that the Mcm:GFP constructs are not wild type for Mcm function and that Rad3 activity is required for viability in *cdc10<sup>ts</sup> mcm:GFP* background. We are also addressing the question whether other checkpoint proteins are required for the pre-RC loading delay.

An interesting possible mechanism of Rad3 action is chromatin modification, a molecular change suggested as being an important component of checkpoint activation (Koundrioukoff et al., 2004). In the nucleus, DNA is wrapped around histones to form nucleosomes and other higher-order compact chromatin structures. The nucleosomes also act as a natural physical barrier, where chromatin remodelling complexes enable highly



regulated access to DNA sequences. To overcome the physical barrier, post-translational modifications on the histone tails are required to regulate biological processes, such as transcription, replication and DNA repair (Kouzarides, 2007). One report suggest that one histone modification contributes to the checkpoint response by recruitment of Crb2 to damaged DNA (Sanders et al., 2004). Rad3 could possibly be involved in some kind of chromatin modifications that affect the loading of pre-RC onto the chromatin. Evidence for a role of chromatin remodelling in DNA replication has also emerged. It is suggested that chromatin remodelling may be required for ORC to bind and function efficiently. ATP-dependent chromatin remodelling required to move nucleosomes around the replication origin either to unmask the ORC-binding site or to configure the nucleosomes around the ORC-binding site to precise positions, allowing complexes are candidates for achieving such nucleosomal movements. If indeed chromatin remodelling complexes are needed to enhance ORC binding or function, these complexes themselves need to be recruited to the replication origin. One mechanism could be through binding to ORC directly or by interacting with other replication initiating factors, such as Cdc18 and Cdt1 (Falbo and Shen, 2006).

#### ***The novel pathway might represent a general mechanism coupling cell growth and the cell cycle***

Our finding that Gcn2 can regulate the cell cycle is novel and suggests a possible link between general growth (translation) and cell cycle progression (G1/S transition). Such a link is logical and has been hypothesized to exist because there must be some kind of coupling between cell growth and cell cycle progression, in order to maintain stable growth and proliferation (Jorgensen and Tyers, 2004). However, the nature of this coupling has not been established in any organism. Cell proliferation is primarily regulated by available energy sources and it is reasonable for the cell to have molecules that sense nutrition status to communicate with high-energy-requiring processes such as translation and the cell cycle machinery. Proteins reported to interact both with the translation machinery and the pre-RC components, such as Yph1 and Noc3 are candidates for such a role (Du and Stillman, 2002; Zhang et al., 2002). It is not trivial to identify molecules that serve as a link between cell proliferation, DNA replication,

ribosome biogenesis, and translation. One fundamental restriction on the growth of cells is their translational capacity and hence the number of ribosomes they possess. If a protein correlated with growth is lacking the consequence could be reduced accumulation of mass. Naturally, the cell cycle transitions will be affected due to decreased rate of protein synthesis and consequently necessary mass accumulation is delayed. It does not necessarily mean that the encoded protein has a function in regulating the cell cycle during growth of wild type cells (Jorgensen and Tyers, 2004). Given the central role of ribosome biogenesis in growth it is not surprising that it has a major role in controlling cell cycle progression. The TOR kinase is commonly known to regulated ribosome biogenesis and other aspects of translation and is referred to as the central controller of growth in mammalian cells. Therefore we explored the role of Tor in the G1 checkpoint, as discussed under project 3.

#### ***Strategies to delay entry into S phase in response to DNA damage in G1***

Most eukaryotic cells delay entry into S phase when the DNA is damaged in G1. Replicating the damaged DNA is not advantageous for the cell, it is better to deal with damages before the cells enter S phase. Delayed progression into S phase upon DNA damaged is believed to allow expression of genes that facilitate repair and give the cells time to repair the DNA damage (Huberman, 1999). Such G1 checkpoints exist in budding yeast (Siede, 1995), *Xenopus* (Costanzo et al., 2000) and in human cells (reviewed in Zhou and Elledge, 2000).

A G1 checkpoint operating in budding yeast has been reported to target Swi6 (the equivalent in *S. pombe* is Cdc10), thereby delaying the transcription of the target genes when the G1/S checkpoint is active (Sidorova and Breeden, 1997). We found that transcription of the *cdc10*-dependent genes *cdt1* and *cdc18* is not delayed after UV irradiation in the *cdc10* block-and-release experiments. The same was observed in germinating spores; *cdc10*-dependent genes were transcribed on time, independently of UV irradiation. The G1-delay we observed does not target the transcription of S-phase-

specific genes, therefore the G1/S delay is not operating via the same pathway as described in budding yeast.

G1 checkpoints operating in mammalian cells either delay the transcription programme required for S phase entry or inhibit CDK activity (Bartek and Lukas, 2001; Lukas et al., 2004). CDK inhibition delays the loading of Cdc45 (an essential replication factor) onto the replication origins subsequent to the pre-RC formation. CDK inhibition also blocks activation of the E2F transcription factor, which is required for activation of S phase genes in mammalian cells. In fission yeast Cdc2 is not phosphorylated during the G1 delay, nor is the transcription program required for S phase delayed.

The translation of G1 cyclin genes has been shown to be repressed under certain stress conditions both in budding yeast (Gallego et al., 1997; Philpott et al., 1998; Polymenis and Schmidt, 1997), fission yeast (Grallert et al., 2000) and in human cells (Hamanaka et al., 2005), thereby linking translation to regulation of progression through G1 phase. In the G1/S checkpoint presented here, the cell cycle delay occurs before any cyclin is required, making the *S. pombe* G1 cyclin, Cig2, not likely to be the major target of the checkpoint. However, a recent publication (Geng et al., 2007) shows that mammalian cells Cyclin E (the G1/S cyclin) performs a function in forming the pre-RC in a manner that is, surprisingly, independent of its kinase partner. Therefore the possibility of Cig2 to have an unknown function independent of Cdc2 will be investigated in *S. pombe*.

Interestingly, a DNA-damage-induced checkpoint that operates via Cdt1 proteolysis in fission yeast has recently been discovered (Ralph et al., 2006). The Cdt1 proteolysis is checkpoint Rad independent and Cdt1 turnover is dependent on the degradation proteins Ddb1 and Cdt2. Involvement of Ddb1 and Cdt2 in Cdt1 proteolysis has also been reported in *Drosophila*, *Xenopus* and mammalian cells (Higa et al., 2003; Jin et al., 2006). Germinating spores delay with low levels of Cdt1 after UV irradiation. It will be interesting to investigate whether the low levels of Cdt1 observed in irradiated germinating spores is due to reduced translation or increased degradation.

***The novel G1 checkpoint is likely to be conserved through evolution***

It has been found that the Gcn2 kinase is activated by UV irradiation of human cells, followed by eIF2 $\alpha$  phosphorylation and inhibition of translation (Deng et al., 2002; Wu et al., 2002). Phosphorylation of eIF2 $\alpha$  was also observed after UV irradiation of budding yeast cells in G1 (our unpublished data). The cell cycle effect of Gcn2 activation has not been shown but we think it is likely that the Gcn2-dependent checkpoint pathway operating in G1 is conserved through evolution, as is the case for other checkpoints.

### Project 3

#### **Tor proteins are not required for regulating eIF2 $\alpha$ phosphorylation in response to UV irradiation**

Cell growth is tightly coupled to nutrient availability, the energy status of the cell and in the case of higher eukaryotes, to the presence of growth factors. TOR integrates these inputs to control cell growth. Yeast cells harbour two Tor genes, *tor1* and *tor2*, whereas other eukaryotes contain only one *tor* gene. Two structurally and functionally distinct multiprotein TOR complexes are identified in budding yeast and mammals. These TOR complexes regulate translation through modulation of the activity of the cap-binding complex eIF4 and via regulation of ribosome biogenesis. Given that the Tor kinases are well known regulators of translation in response to various stresses, it was of great interest for us to investigate whether Tor1 and Tor2 in fission yeast have any role in the UV-induced eIF2 $\alpha$  phosphorylation.

Tor2 function in *S. pombe* is essential and therefore a temperature-sensitive mutant has been used to elucidate the role of Tor2. The Tor1 deletion mutant is viable, but the strain is sterile. We found that UV-induced eIF2 $\alpha$  phosphorylation occurs in cells lacking Tor1 or Tor2 as well as in the *tor1 $\Delta$ tor2<sup>ts</sup>* double mutant, indicating that Tor activation is not required to reduce the overall translation rate after UV irradiation.

Surprisingly, we observed that eIF2 $\alpha$  was phosphorylated in the *tor2<sup>ts</sup>* mutant when growing at its restrictive temperature, even without any further treatment. This result suggests that Tor2 might also control translation initiation by regulating the phosphorylation status on eIF2 $\alpha$  phosphorylation. Under normal conditions, Tor2 is active in order to promote translation and keeping eIF2 $\alpha$  unphosphorylated. However, in response to stress Tor2 might slow down the overall biosynthesis through phosphorylation of eIF2 $\alpha$ .

How Tor2 regulates the phosphorylation status on eIF2 $\alpha$  is at present not known. It can be speculated that Tor2 keep eIF2 $\alpha$  unphosphorylated by inactivating eIF2 $\alpha$  kinases, or

activating phosphatases. We have investigated this by mean of the *tor2<sup>ts</sup> gcn2 hri1 hri2* quadruple mutant. Surprisingly, eIF2 $\alpha$  is phosphorylated in the quadruple mutant as efficiently as in the *tor2<sup>ts</sup>* strain. This strongly suggests that there is another, unidentified eIF2 $\alpha$  kinase in fission yeast. Bioinformatics was employed in the search for other possible eIF2 kinases (Knutsen, J.H, personal communication). The sequences of eIF2 $\alpha$  kinases from several organisms were aligned, with the kinase domain deleted. A short region outside the kinase domain was found to be conserved among the aligned eIF2 $\alpha$  kinases and a BLAST search against the *pombe* database was performed using this query sequence. The search produced three hits with high similarity to the short conserved region; Hri1, Hri2 and a third protein with unknown function, called Psk1. The fact that the Hri kinases were identified in the search verifies the approach, although it is interesting that Gcn2 did not have a high score in the BLAST search. It is promising that Psk1 has a kinase domain and we shall explore whether this putative kinase can phosphorylate eIF2 $\alpha$ .

In response to starvation, protein synthesis is repressed through phosphorylation of eIF2 $\alpha$  performed by Gcn2. We were curious about whether this phosphorylation is influenced by any of the Tor proteins. We found that Tor2, Tor1 and Gcn2 are all required for eIF2 $\alpha$  phosphorylation in cells starved for leucine. Thus, the activation of Gcn2 appears to be different in leucine-starved and in UV-treated cells, since it is Tor-dependent in the former but not in the latter situation.

In summary, we have shown that Tor can affect translation initiation via regulating eIF2 $\alpha$  phosphorylation under some circumstances. The UV-induced eIF2 $\alpha$  phosphorylation does not depend on Tor. Revealing the molecular basis of how and when Tor regulates translation initiation through eIF2 $\alpha$  phosphorylation and how this function correlates with Tor's roles in ribosome biogenesis and controlling the activity of eIF4 awaits further analysis.

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